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13. ABSTRACT (Maximum 200 Words)
Fibroblast Growth Factor-2 (FGF-2) is a biologic cytokine with a major role in tumorigenesis and angiogenesis, as well as in organogenesis and neuroprotection. FGF-Binding Protein 1 (BP1) has been suggested to bind, protect, and present FGF-2 to its receptor on the cell surface. Studies of this protein thus far have mainly relied on transfection or ribozyme-targeting protocols to evaluate its functional activity. We produced and purified two recombinant BP1 proteins, in prokaryotic and eukaryotic systems. We use recombinant BP1 to study the binding characteristics and functional activity of BP1. We show that recombinant BP1 can reversibly bind radiolabeled FGF-2, and that this binding can be inhibited by high doses of FGF-1, heparin, heparan sulfate, and pentosan polysulfate (PPS), a heparinoid presently in clinical trials. We demonstrate that recombinant BP1 can positively modulate FGF-2 activity in models of anchorage-independent growth and angiogenesis. We have used peptide phage display technology to investigate peptide sequences which may offer superior binding characteristics to FGF-2 or BP1. We are presently investigating the ability of BP1 to affect the role of FGF-2 in neuroprotection models. Additionally, we are investigating the intracellular signaling cascade of BP1-modulated FGF-2 activity. We conclude that BP1 reversibly binds and positively modulates the functional activity of FGF-2 in multiple models of FGF-2 mediated events.

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FOREWORD

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Ali Al-Uttan   _July 31, 2000_
PI - Signature   Date

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Introduction

Fibroblast Growth Factors (FGF's) are a major family of proteins which mediate
physiologic processes including angiogenesis, tumorigenesis, and neuroprotection. The
extracellular activity of these proteins, especially that of FGF-2, the major FGF studied,
is not well-correlated with their concentrations, largely because FGF's are sequestered in
the extracellular matrix by binding to heparan sulfate proteoglycans. A novel protein has
been described which binds, protects, and presents FGF-2 and possibly other FGF's to
their extracellular cell-surface receptor. We hypothesize that it is the regulation of this
protein, FGF-Binding Protein (BP1), which controls the physiologic concentration of
FGF-2. We produced two recombinant BP1 proteins with which we have studied the
binding properties and biology of BP1. We have confirmed that BP1 specifically and
reversibly binds FGF-2 as well as FGF-1. Additionally, we have been able to examine
the role of BP1 in models of angiogenesis and anchorage independent growth. We have
used phage display technology to look for peptides which bind BP1 or FGF-2 with
superior specificity and affinity. We are presently also examining the ability of BP1 to
modulate the neuroprotective role of FGF-2. In summary, we have demonstrated some of
the binding characteristics and kinetics of BP1, and found that it can positively modulate
the angiogenic and tumorigenic properties of FGF-2.

Body

BP1 was first described in 1991 as a binding protein for FGF-1 and FGF-2 and was shown to bind these proteins. This initial report presented data which suggest that BP1 modulates the activity of FGF-1 and FGF-2, and that this inhibition, as well as the binding, can be abolished by heparin.

A series of studies in our laboratory have demonstrated that BP1 plays a crucial role physiologically in embryogenesis and pathologically during epithelial carcinogenesis. BP1 expression at the mRNA and protein levels are highly regulated through these processes. Most significantly, studies using transfection and ribozyme-targeting of BP1 mRNA indicate that BP1 might actually be rate-limiting in angiogenesis and carcinogenesis in a number of *in vitro* and *in vivo* models, proposing BP1 as the "angiogenic switch" for human cancer.

In addition to human BP1, homologues have been identified in mouse and bovine. Having identified BP1 as a component of bovine prepartum mammary gland secretions, Lametsch et al. found that bovine BP1 shares all 10 conserved cysteine amino acids with mouse and human BP1 homologues, suggesting similar tertiary structure across species, as well as raising the possibility that this protein plays crucial roles in physiologic and pathologic processes in mammalian biology.

Heparin-like glycosaminoglycans (HLGAG's) and heparin bind FGF-2 and are present either as cell-surface bound molecules or in the extracellular matrix. Binding of FGF-2 to the basement membrane is due to the HLGAG's contained therein. Moreover, binding of FGF-2 to its high-affinity receptors requires cell-surface HLGAG's, and several of FGF-2's functional effects appear to be dependent on presentation through heparin. Heparin and heparin-like molecules thus function in the sequestration of FGF-2 in the extracellular matrix and its presentation to its receptors on the cell surface.

Our research is the first systematic analysis of the direct effects of recombinant BP1 in *in vitro* and *in vivo* systems. Our data confirm earlier studies by Wu et al. that BP1 binds FGF-2 in a specific, reversible manner, as well as earlier studies by our laboratory which suggested that BP1 serves as the angiogenic switch in human cancer.

To evaluate the direct effects of BP1 on FGF-2 dependent activities, we produced two recombinant BP1 proteins, one in each prokaryotic and eukaryotic systems. A GST-BP1 fusion protein was made in bacterial cells, and a bilaterally histidine-tagged BP1 recombinant protein (His-BP1) was made through baclovirus infection of Sf-9 insect cells. To assess if these proteins were purified to homogeneity we ran them on polyacrylimide gel under denaturing conditions and visualized with coomassie blue staining. A primary polyclonal antibody generated against a peptide sequence of BP1 recognized BP1 protein as a discrete band of appropriate molecular size in a Western Blot of this gel.

We next sought to assess whether these recombinant proteins, purified using denaturing treatment, still retained the ability to bind FGF-2. We used a binding assay where recombinant BP was bound to wells, blocked, and radiolabeled ^{125}I-FGF-2 was added at increasing concentrations, incubated for 60 minutes, and then washed off. Radioactivity remaining in the wells was quantified, reflecting binding of ^{125}I-FGF-2 to recombinant BP1. This was compared with control wells where recombinant BP1 was not pre-adsorbed to wells.

Our binding studies showed that both recombinant proteins bound ^{125}I-FGF-2 in a dose-dependent, saturable fashion. These initial studies were repeated numerous times to determine optimal binding conditions for both recombinant BP1 proteins. Since His-BP1 showed superior binding to GST-BP1, and due to the smaller molecular modification of His-BP1 (only six histidine residues on each side, compared with a bulky 20 kD GST fusion component), we decided to proceed further studies exclusively with the His-BP1 recombinant protein.

To document specificity of His-BP1 binding to ^{125}I-FGF-2, we ran competition experiments where non-radiolabeled FGF-2 was added to the wells simultaneously with ^{125}I-FGF-2. Our experiments demonstrated saturable binding of FGF-2 to His-BP1, as the non-radiolabeled FGF-2 was able to fully compete off the ^{125}I-FGF-2 within the concentration range expected. Similarly, since His-BP1 was described to bind FGF-1 as well, we decided to test whether binding of FGF-1 to His-BP1 affected its affinity for FGF-2. A competition experiment was designed where FGF-1 and ^{125}I-FGF-2 were simultaneously added to wells pre-adsorbed with His-BP1 (as well as control wells without pre-adsorbed His-BP1). Our studies indicated that FGF-1 could compete off ^{125}I-FGF-2 binding to His-BP1 as effectively and in the same concentration range as non-radiolabeled FGF-2, suggesting the possibility that BP1 binds both proteins at the same site on BP1.

Since both BP1 and FGF-2 bind heparin and heparan sulfate (both were initially described as heparin-binding proteins), we decided to evaluate the effect of heparin and heparan sulfate in this competition assay design. Since heparin and heparan sulfate bind both molecules, we wanted to know whether this could lead to a "bridge-like" conformation with heparin holding both proteins simultaneously (and thus increasing radioactive binding), or whether heparin would bind to one of the two molecules and change its conformation in such a way as to decrease binding of ^{125}I-FGF-2 to His-BP1 (and thus decrease radioactive binding). Our studies found that increasing concentrations of heparin or heparan sulfate caused decreased binding of ^{125}I-FGF-2 to His-BP1, maximally to baseline binding. This suggests that the presence of heparin or heparan sulfate, which are found in the extracellular matrix of biologic systems, could serve to raise the threshold of binding of BP1 to FGF-2.

Pentosan polysulfate (PPS) is a heparanoid molecule presently in clinical trials for its antineoplastic activity. Two previous studies clearly demonstrated that the pro-neoplastic effect of FGF is neutralized with PPS, suggesting a mechanism for its anti-neoplastic activity. We therefore used PPS in our competition assay design to evaluate its role in the interaction between FGF-2 and BP1. Our experiments showed that PPS inhibits binding of ^{125}I-FGF-2 to His-BP1 in a dose-dependent manner, with maximal inhibition to baseline at a concentration of PPS that is 100-fold lower than those of heparin or heparan sulfate needed to achieve full inhibition of binding. These results lend support to the hypothesis that at least some of PPS's anti-neoplastic activity is due to its interference with the binding of BP1 to FGF-2.

After these cell-free *in vitro* studies documented that our recombinant BP1 retained the capacity to bind FGF-2 and interact with related molecules, we wanted to evaluate the effects of direct addition of BP1 to biologic systems. We selected two *in vitro* models to study BP1: soft agar assay and endothelial proliferation assay.

Soft agar assay is a model of anchorage-independent growth, a phenotypic characteristic of tumor cells following progression to malignancy. SW13 adrenal adenoma cells were cultured in an agar bilayer and exposed to different treatment combinations of FGF-2 and His-BP1. Following eight days of growth, colony number and size was evaluated, as increased numbers of larger colony sizes correlates with anchorage-independent growth phenotype. We were able to repeatedly achieve a dose-response curve with FGF-2 alone, and we proceeded to test varying concentrations of His-BP1 added to the different FGF-2 concentrations. Our experimental results, representative samples of which are presented in Figure 3A, demonstrate that His-BP1 can significantly increase the number of colonies exhibiting anchorage-independent growth, both with and without addition of exogenous FGF-2. The finding of increased colony growth due to addition of His-BP1 without addition of exogenous FGF-2 is explained by the endogenous production of FGF-2 by SW13 cells themselves. This is the first evidence of the direct effect of BP1 on biologic systems.

Endothelial proliferation assay is a model of angiogenesis, where HUVEC's are plated on wells and then incubated in the presence of varying concentrations of FGF-2 and His-BP1. Following five days, cell count is assayed using a reagent (WST-1) which changes color through a mitochondrial enzymatic reaction. Since we were able to demonstrate a dose-dependent increase in HUVEC proliferation rate with FGF-2, we wanted to examine the effect of direct addition of His-BP1 to this system. Our studies showed a moderate yet consistent positive modulation of the effects of FGF-2 on endothelial proliferation. The greatest benefit from His-BP1 occurred at exogenous FGF-2 concentrations of 1 ng/ml; this contrasts with the maximal modulation at 0.1 ng/ml exogenous FGF-2 seen in soft agar assay. Additionally, His-BP1 did not affect endothelial proliferation when no exogenous FGF-2 was added. These two findings are consistent with evidence that HUVEC's do not produce endogenous FGF-2.

Our laboratory wanted to examine the role of direct addition of BP1 in an *in vivo* system. We decided to use the chorioallantoic membrane (CAM) assay to examine this relationship, as this is a well-established *in vivo* model of angiogenesis. Sterile Whatmann filter papers are cut into 8 mm disks and applied between blood vessels on the chorioallantoic membrane of five-day-old chick embryos. Various concentrations of FGF-2 and His-BP1 are added to the disk, and degree of angiogenesis is assessed by blinded observer at 0, 12, 24, and 36 hours. Degree of angiogenesis is scored from 1 (minimal) to 4 (maximal), reflecting new blood vessel formation. Our studies demonstrate that addition of either FGF-2 or His-BP1 lead to significantly enhanced angiogenesis, yet maximal angiogenesis occurs with addition of both FGF-2 and His-BP1. His-BP1 addition without exogenous FGF-2 is able to increase angiogenesis because CAM contains endogenous FGF-2. A time course study demonstrates that the addition of His-BP1 alters the temporal dynamics of angiogenesis by inducing earlier blood vessel formation, up to a maximal degree which is eventually reached by FGF-2 addition.

Key Research Accomplishments

--We produced and purified two recombinant FGF-Binding Protein (BP1) proteins in procaryotic and eucaryotic systems;

--we determined the binding characteristics and kinetics of BP1 binding to FGF-2, FGF-1, heparin sodium, heparan, and Pentosan Polysulfate (PPS);

--we demonstrated that recombinant BP1 added exogenously to *in vitro* models of angiogenesis (endothelial proliferation assay) and tumorigenesis (anchorage independent growth—soft agar assay) can positively modulate the effects of FGF-2;

--we demonstrated that recombinant BP1 added exogenously to an *in vivo* model of angiogenesis (chorioallantoic membrane assay) can positively modulate the effects of FGF-2.

Reportable Outcomes

(Although there are no Reportable Outcomes at this time, we are presently in the process of submitting two papers to peer-reviewed journals to report the results of our work to date.)

Conclusions

We demonstrate in a cell-free system that BP1 binds FGF-2 reversibly, that this binding can be disrupted in a dose-dependent manner by FGF-1, heparin, heparan sulfate, and PPS. Moreover, structural analysis using mass spectroscopy confirm binding of BP1 and FGF-2 at the biochemical level. *In vitro* studies show that BP1 positively modulates the tumorigenic and angiogenic effects of FGF-2 at physiologic concentrations of both proteins. We found in an *in vivo* model of angiogenesis, the CAM assay, that maximal stimulation of angiogenesis required the addition of BP1.

Both heparin and BP1 have been shown to bind FGF-2 in the extracellular matrix, However, the relationship between the roles of heparin and BP1 remains unclear. While it is established that FGF-2 is sequestered in the extracellular matrix predominantly bound to heparin moieties in the basement membrane, the solubilization of FGF-2 appears to be due to the arrival of either heparinases or of BP1. The added function of protection is afforded by BP1 and heparin, whereas heparinases and other proteases may serve equally to digest FGF-2 *in vivo*.

It therefore appears as though BP1 and heparin-like molecules act in surrogate roles in the binding, protection, and possibly even the presentation of FGF-2. It is consistent with all reported studies on BP1 that it serves, when present, to shuttle FGF-2 from the heparin in the extracellular matrix to that on the cell surface, therefore ushering in the enhanced activity of FGF-2-dependent functions, as detected by our various assays. This model is well-supported by hybridization studies which demonstrate that BP1 mRNA expression is highly regulated and its presence is strongly correlated with the effects of FGF-2-dependent activity. Whereas most adult tissues have no basal BP1 mRNA production, a pre-malignant focus can, by production of BP1, mobilize the extracellular, heparin-bound FGF-2 and thus switch on the angiogenic machinery essential for malignant growth.

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Appendices

None.